United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 217

Supplemental Assay Method for Potency Testing of Tetanus Toxoid by ELISA

Date: **June 7, 2006**

Number: SAM 217.02

Supersedes: BBSAM0217.01, November 5, 1999

Standard Requirement: 9 CFR, Part 113.114

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Supplemental Assay Method for Potency Testing of Tetanus Toxoid by ELISA

Table of Contents

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1. Introduction

This Supplemental Assay Method (SAM) is used to determine the potency of tetanus toxoid as prescribed by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.114. Guinea pigs are vaccinated and bled 6 weeks later. The serum is assayed for antitoxin content by indirect Enzyme-Linked Immunosorbent Assay (ELISA).

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Orbital shaker

2.1.2 Test tube mixer, vortex-type

2.1.3 Incubator, 35°- 37°C, with cooling capacity

2.1.4 Refrigerator, 2°- 7°C

2.1.5 Freezer, -20°C or lower

2.1.6 Centrifuge with horizontal centrifuge head that accommodates blood collection tubes

2.1.7 Microplate washer/aspirator

2.1.8 Microplate reader

2.1.9 Computer with software that performs linear regression analysis

2.1.10 Micropipettors, 20-µL, 100-µL,and 1000-µL

2.1.11 Multichannel micropipettor, 50- to 300-µL

2.2 Supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Tips for micropipettors

MW 12 Jun 06

2.2.2 Flat-bottom 96-well microtitration plates (Nunc™ F96 maxisorb)

2.2.3 Syringes, needle-locking

2.2.4 Needles, 23-gauge and gauge approved for blood collection

2.2.5 Blood collection tubes

2.2.6 Pipettes, assorted sizes

2.2.7 Tubes and flasks (with caps), various sizes to make reagent dilutions

2.3 Chemicals and reagents

Equivalent chemicals or reagents may be substituted for any brand name listed below.

2.3.1 Water, deionized or distilled, or water of equivalent purity

2.3.2 Sodium chloride (NaCl)

2.3.3 Nonfat dry milk

2.3.4 Sodium bicarbonate ($NaHCO_3$)

2.3.5 Sodium carbonate (Na_2CO_3)

2.3.6 Sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2.3.7 Sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)

2.3.8 Sodium hydroxide (NaOH), 5M

CAUTION! Toxic and Hazard Review (THR) of liquid sodium hydroxide: Poison by intraperitoneal route. Moderately toxic by ingestion. Mutagenic data. A corrosive irritant to skin, eyes, and mucous membranes. This material, both solid and in solution, has markedly corrosive action upon all body tissue causing burns and frequently deep ulceration, with ultimate scarring. Mist, vapors, and dusts of the compound cause small burns, and contact with the eyes rapidly causes severe damage to the delicate tissue.

2.3.9 Polyoxyethylene-sorbitan monolaurate (Tween 20)

12 June 06

2.3.10 Goat anti-guinea pig IgG (H+L) conjugated to horseradish peroxidase, Jackson Immunoresearch Laboratories (Catalog #106-035-003)

2.3.11 2,2'-azino-di-(3-ethylbenzthiazoline-6, sulfonate) [ABTS] peroxidase substrate, Kirkegaard and Perry Laboratories (Catalog #50-62-00)

2.3.12 *Clostridium tetani* toxin, current lot, provided by the Center for Veterinary Biologics (CVB)

2.3.13 *C. tetani*-negative guinea pig serum, current lot, provided by the CVB

2.3.14 *C. tetani*-positive guinea pig serum, (5 AU/mL), current lot, provided by the CVB

2.4 Test animals

Guinea pigs, 450-550 g. A group of 10 guinea pigs consisting of an equal number of males and females are required for each serial. Select guinea pigs that are healthy, free of external parasites, and have an unblemished hair coat. Do not use pregnant guinea pigs.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need to have a working knowledge of the use of general laboratory chemicals, equipment, and glassware; and have specific training and experience in the safe handling of clostridial toxins. Personnel need specific training in the care and handling of laboratory guinea pigs.

3.2 Preparation of equipment/supplies

3.2.1 Operate all equipment according to the manufacturers' instructions.

3.2.2 Use sterile supplies.

12 Jun 06

3.3 Preparation of reagents

3.3.1 Wash solution

1. Add 17 g of NaCl, 0.44 g $NaH_2PO_4 \cdot H_2O$, 2.38 g Na_2HPO_4, and 1.0 mL Tween 20 to 2 L of deionized water. Mix until completely dissolved.

2. If necessary, adjust the pH to 7.2 with 5 M NaOH.

3. Autoclave at ≥120°C for 30 to 35 minutes.

4. Store at 20°- 25°C for up to 6 months.

3.3.2 Antigen coating buffer

1. Add 0.795 g Na_2CO_3 and 1.465 g $NaHCO_3$ to 500 mL of deionized water. Mix until completely dissolved.

2. If necessary, adjust the pH to 9.6 with 5 M NaOH.

3. Store the buffer at 2°- 7°C for no longer than 1 week.

3.3.3 Blocking solution

1. Add 2.5 g of nonfat dry milk to 250 mL wash solution (**Section 3.4.1**). Mix until dissolved. If necessary, heat just enough to dissolve milk.

2. Adjust the pH to 7.9 with 5 M NaOH.

3. Store the solution at 2°- 7°C for no longer than 1 week.

3.3.4 1% Skim Milk (SM) diluent

1. Add 5 g of nonfat dry milk to 500 mL wash solution (**Section 3.4.1**). Mix until completely dissolved, heating if necessary.

2. Add 0.25 mL of Tween 20 and stir until evenly distributed in the mixture.

3. Adjust the pH to 7.2 with 5 M NaOH.

4. Store the diluent at 2°- 7°C for no longer than 1 week.

JMW 12 Jun 06

Supplemental Assay Method for Potency Testing of Tetanus Toxoid by ELISA

4. Performance of the test

4.1 Vaccination of test animals

4.1.1 Check the container label and the manufacturer's Outline of Production of each product to be tested for identity and recommended field dose. The dose for guinea pigs is 0.4 of the largest dose recommended on the product label.

4.1.2 Mix the product thoroughly before filling syringes. Vaccinate each guinea pig subcutaneously (using a 23-gauge needle) in the ventral thoracic area.

4.2 Blood sample collection and serum preparation

4.2.1 Collect a 10 mL blood sample from each guinea pig 42 to 45 days after vaccination. Use a blood collection protocol approved by the Institutional Animal Care and Use Committee.

4.2.2 Centrifuge the blood from each animal separately at 1000 x *g*, and dispense the serum into individually marked tubes.

4.2.3 Pool equal portions of serum, but not less than 0.5 mL from each guinea pig, and store it at 2°- 7°C for up to 7 days. The serum pool must be made from all guinea pigs that survive the vaccination and must contain serum from at least 8 animals. If the test will not be conducted for at least 7 days after the date of blood collection, store serum at -20°C or lower prior to use.

4.3 ELISA Procedure

4.3.1 Coat the microtiter plate with tetanus toxin

1. Dilute *C. tetani* toxin in antigen coating buffer to the working dilution specified in the reagent data sheet for the lot of toxin being used.

CAUTION! Accidental parenteral inoculation and ingestion of tetanus toxin are the primary hazards to laboratory personnel. It is unknown if the toxin can be absorbed through mucous membranes; consequently, the hazards associated with aerosols and droplets remain unclear.

2. Dispense 100 µL of diluted toxin into each well of a 96-well microtitration plate. Cover the plate. Incubate the plate overnight in a humidified chamber at 20°- 25°C on an orbital shaker operating at 50-70 revolutions per minute (rpm).

12 Jun 06

3. At the end of the incubation period, invert the plate to empty any unbound toxin into a discard container and blot on absorbent disposable toweling to remove any residual toxin. Autoclave all toxin reagent prior to disposal.

4.3.2 Block the microtitration plate

1. Fill each well of the microtitration plate with 250-300 µL blocking solution.

2. Cover the plate and incubate it in a humid atmosphere at 35°- 37°C for 120 to 140 minutes while mixing on an orbital shaker (50-70 rpm).

Note: Use an incubator with refrigeration capability or the heat from the shaker will cause the temperature to increase to an unacceptable level.

3. Invert the plate and blot on absorbent toweling to remove the excess blocking solution. If not proceeding directly to **Section 4.3.3**, cover the plate and place it in a plastic bag. Store the plate in an inverted position at 2°- 7°C for no longer than 1 week.

4.3.3 Add the serum samples

1. Wash the plate 5 times using 250-300 µL of wash solution per well. Invert the plate and tap it on absorbent material to remove all traces of residual wash solution.

2. Make an initial 1:4000 dilution of all sera (positive control, negative control, and each test serum pool) in SM diluent as shown in **Figures 1** and **2**. This working dilution falls within the dynamic response range of the assay.

3. Make additional incremental dilutions of the positive guinea pig serum as illustrated in **Figure 1**. These dilutions will be used to create a standard response curve against which the test sera are compared.

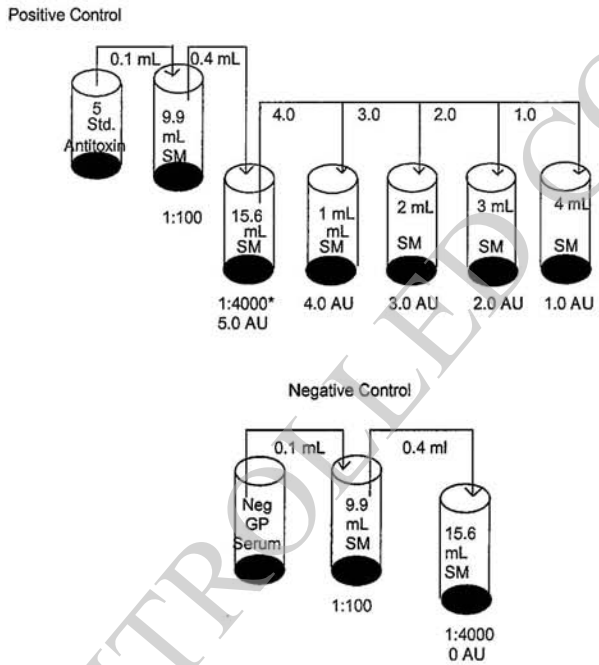
4. Add 100 µL of SM diluent to each well in column 1. Use these wells to blank the microplate reader. Do not use the remaining outside wells because they may provide inconsistent readings.

12Jun06

Supplemental Assay Method for Potency Testing of Tetanus Toxoid by ELISA

5. Add 100 µL of each diluted control serum to each of 3 replicate wells. Add 100 µL of each test serum pool to each of 6 replicate wells. An example plate diagram is shown in **Figure 3**.

6. Cover the plate and incubate it in a humid atmosphere for 16 to 24 hours at 2°- 7°C while mixing on an orbital shaker (50-70 rpm).



*Positive guinea pig serum diluted 1:4000 represents 5.0 AU/mL on the standard curve. Negative serum represents 0 AU/mL

Figure 1. Dilution of the Positive Guinea Pig Serum and Negative Guinea Pig Serum

JMW 12Jun06

Supplemental Assay Method for Potency Testing of Tetanus Toxoid by ELISA

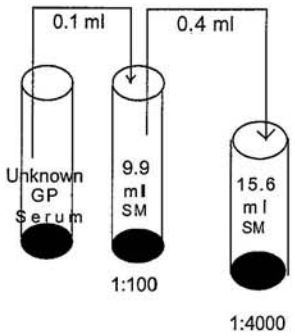
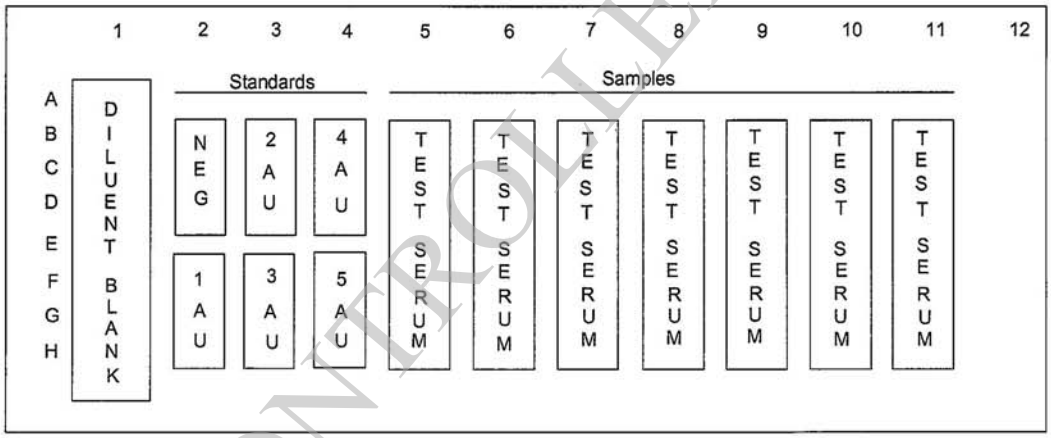


Figure 2. Dilution of the Unknown Guinea Pig Serum

Figure 3. Example Plate Diagram



4.3.4 Add the conjugate

1. Wash the plate as described in **Section 4.3.3(1).**

2. Dilute the conjugate with SM diluent to the optimized working dilution for the lot (1:10,000 is suggested if that dilution falls within the manufacturer's recommended ELISA range).

3. Add 100 µL of diluted conjugate to each well of the plate.

4. Cover the plate and incubate it in a humid atmosphere at 35°- 37°C for 120 to 140 minutes while mixing on an orbital shaker (50-70 rpm).

JMW 12Jun06

4.3.5 Add the substrate

1. Ten to 25 minutes before use, follow the manufacturer's instructions to prepare 12 mL of ABTS substrate. Allow the substrate to warm to 20°-25°C. Protect it from light.

2. Wash the plate as described in **Section 4.3.3(1)**.

3. Dispense 100 µL of substrate solution into the wells of the plate.

4. Cover the plate and incubate it at 20°- 25°C for 15 to 25 minutes while mixing on an orbital shaker (50-70 rpm). Ideally, the incubation shoud be terminated when the absorbance of the wells containing the 5 AU/mL standard is approximately 1.0.

4.3.6 Read the test plate

Read the absorbance of the ELISA plate at 405/490 nm.

4.4 Calculation of results

4.4.1 Perform a linear regression analysis using data (antitoxin unit concentration vs. absorbance) from the control sera (0-5 AU/mL). Determine the slope, correlation coefficient, and y-intercept of the resultant dose-response line (i.e., standard curve).

4.4.2 Calculate the potency (AU/mL) of the test serum pool by fitting the average absorbance of the test serum pool to the line defined in **Section 4.4.1**.

$$\text{Titer (AU/mL) of test serum pool} = \frac{\text{Absorbance of test serum pool - y intercept}}{\text{Slope}}$$

5. Interpretation of the test results

5.1 For a valid test, a correlation coefficient of at least 0.985 and a slope of at least 0.1 must be achieved. Invalid tests may be repeated.

5.2 If the antitoxin titer of the serum pool is at least 2.0 AU/mL, the test serial is satisfactory.

5.3 If the antitoxin titer of the serum pool is <2.0 AU/mL and the test serial is not retested, it shall be declared unsatisfactory. For serials in which the serum pool contains

<2.0 AU per mL, the individual component sera from the pool may be tested by the assay described above, using the following criteria:

5.3.1 If at least 80% of a minimum of 8 individual serums have an antitoxin titer of at least 2.0 AU per mL, the serial is satisfactory.

5.3.2 If fewer than 80% of the 8 or more individual serums have an antitoxin titer of at least 2.0 AU/mL, the serial may be retested in 10 guinea pigs using the same procedure utilized in the initial test. The antitoxin titer of the pooled serum from the guinea pigs used in the retest shall be averaged with the antitoxin level of the pooled serum from the initial test. If the average of the 2 pools is at least 2.0 AU/mL, the serial is satisfactory. If the average of the 2 pools is <2.0 AU/mL, the serial is unsatisfactory and shall not be retested.

6. Report of the test results

Report results of the test(s) as described by standard operating procedures.

7. References

Code of Federal Regulations, Title 9, Part 113.114, U.S. Government Printing Office, Washington, DC, 2005.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **3.3** This section has been rewritten for clarity.
- **3.4** This section has been deleted and the information is covered in other sections of the document.
- **4.3.3** This section has been rewritten for clarity.
- **4.4** This section has been rewritten for clarity.
- **5.1** Validity requirement for slope was added.

jmw 12 June 06

Supplemental Assay Method for Potency Testing of Tetanus Toxoid by ELISA

- The contact person has been changed to Janet M. Wilson.

JMW 12JUN06